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Studies with the *Plasmodium falciparum* hexokinase reveal that PfHT limits the rate of glucose entry into glycolysis

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ABSTRACT

To characterise plasmodial glycolysis, we generated two transgenic *Plasmodium falciparum* lines, one expressing *P. falciparum* hexokinase (PfHK) tagged with GFP (3D7-PfHK^{GFP}) and another overexpressing native PfHK (3D7-PfHK^{+}). Contrary to previous reports, we propose that PfHK is cytosolic. The glucose analogue, 2-deoxy-D-glucose (2-DG) was nearly 2-fold less toxic to 3D7-PfHK^{+} compared with control parasites, supporting PfHK as a potential drug target. Although PfHK activity was higher in 3D7-PfHK^{+}, they accumulated phospho-[^{14}C]2-DG at the same rate as control parasites. Transgenic parasites overexpressing the parasite's glucose transporter (PfHT) accumulated phospho-[^{14}C]2-DG at a higher rate, consistent with glucose transport limiting glucose entry into glycolysis.

1. Introduction

Malaria is an important parasitic infection caused by members of the genus *Plasmodium*. It is one of the leading causes of morbidity and mortality in the world with 3.1 billion people at risk of being infected [1]. *Plasmodium falciparum* is the most virulent species infecting humans.

Due to the dependence of blood-stage *P. falciparum* on glycolysis for its ATP requirement [2], the pathway represents an attractive target for the development of new and much-needed antimalarials. Glucose entry into the parasite is mediated by the *P. falciparum* hexose transporter, PfHT, a facilitative and saturable transport protein located on the parasite's plasma membrane [3–5]. Glucose transport by PfHT has been validated chemically and genetically as an antimalarial drug target. Previous investigations have shown that PfHT activity can be potently and selectively inhibited by a series of *O*-3 (including compound 3361) and *O*-2 D-glucose derivatives [5–7]. Compound 3361 causes parasite growth inhibition in vivo in *Plasmodium berghei*-infected rodents [6] and, more recently, has been shown to inhibit liver and transmission stages of the murine malarial parasite [8]. Once inside the parasite, glucose is phosphorylated in the cytoplasm by a hexokinase, PfHK, the first enzyme in the glycolytic pathway [9]. Despite its importance to the parasite, little is known about PfHK. The 55.3 kDa protein is encoded by a single intron-less gene located on chromosome 6 [10,11] and has been shown to have a lower affinity for glucose compared with the host erythrocyte hexokinase [9]. Using microscopy data, Olafsson and Certa suggested that PfHK is associated with the parasite plasma membrane (PPM) [12]. This localisation is supported by the presence of a potential hydrophobic membrane anchor sequence at the protein's carboxy terminus [10].

Previous studies have also demonstrated PfHK as a potential drug target. Early experiments showed that two glucose analogues, 2-deoxy-D-glucose (2-DG) and 2-fluoro-2-deoxy-D-glucose (2-FG), inhibited parasite glycosylation and, as a result, parasite growth [13–15]. A subsequent study further described the antiplasmodial effects of 2-DG and 2-FG [16]. The analogues inhibited *P. falciparum* proliferation with increased potency under low glucose conditions, consistent with inhibition of the parasite's metabolism of glucose. Results of subsequent experiments indicated that the analogues exert their antiplasmodial activity, at least partly, by inhibiting glucose phosphorylation. They likely carry this out by acting as PfHK

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substrates (instead of inhibitors) because 2-DG and 2-FG are known to be phosphorylated by PfHK [17] and the human hexokinase, respectively [18]. However, the exact mechanism by which they inhibit parasite growth is still unclear.

In this study we show that PfHK is located throughout the parasite cytosol and that overexpression of PfHK renders the parasites resistant to 2-DG compared with wild-type parasites, consistent with PfHK being the target of 2-DG and, therefore, a potential antimalarial drug target. Furthermore, by measuring 2-DG phosphorylation and accumulation rates in transgenic parasites overexpressing PfHK and PfHT, we present data consistent with the transport of glucose into the parasite by PfHT limiting the rate of glucose entry into *P. falciparum* glycolysis.

2. Materials and methods

2.1. Parasite culture, lysate preparation and transfection

Intraerythrocytic *P. falciparum* parasites (3D7 strain and subsequent transfectants) were cultured, synchronised and isolated as described previously [19] with minor modifications (Supplementary information). Parasite lysates were prepared from saponin-isolated parasites as described previously [20] with some modifications. Isolated parasites were placed in 15 ml tubes and triturated through a 25-gauge SafetyGlide needle (BD) to reduce the risk of needle-stick injuries. Lysates were divided into 150 μl aliquots and stored at −20 °C until required. Parasites were transfected by electroporation as previously described [21] with modifications (Supplementary information).

2.2. Recombinant plasmid construct

PfHK-specific sequence was amplified from *P. falciparum* gDNA via PCR using KOD Hot Start DNA Polymerase (Novagen). The oligonucleotides and the PCR settings are shown in Supplementary information. Restriction enzyme-digested inserts were ligated into pGlux-1 using the Quick Ligation Kit (New England Biolabs). RbCl-competent *Escherichia coli* cells were transformed with the recombinant plasmids as previously described for $CaCl_2$-competent cells [22] and transformants selected using ampicillin. Plasmid DNA sequencing was carried out at the Australian Genome Research Facility (Sydney).

2.3. Confocal microscopy

3D7-PfHK^{GFP}-infected erythrocytes (10–15% parasitaemia, ~4% haematocrit, 5–10 μl) were examined live, and images of the cells captured, with a Zeiss LSM 780 confocal microscope (Carl Zeiss MicroImaging GmbH), at the Centre for Advanced Microscopy, The Australian National University, using a 40 × water immersion or 100 × oil immersion lens.

2.4. Flow cytometry

To determine the percentage of fluorescent 3D7-PfHK^{GFP} parasites, saponin-isolated 3D7 (used to establish level of autofluorescence) and 3D7-PfHK^{GFP} trophozoites were analysed by FACS. Isolated parasites were suspended in standard malaria saline (125 mM NaCl, 5 mM KCl, 25 mM HEPES, 20 mM D-glucose, 1 mM $MgCl_2$, pH 7.1) at a concentration of ~10^5 cells/ml. Measurements of 10 000 cells were taken in duplicate with the following parameters and plotted on a log scale: Forward Scatter: 450 V, Side Scatter: 350 V and Alexa Fluor (GFP fluorescence intensity): 700 V. Data analysis was carried out using FlowJo version 8.8.4 for Mac.

2.5. Protein fractionation and Western blot

Saponin-isolated 3D7-PfHK^{GFP} trophozoites were digitonin permeabilised as described previously [23] with modifications (Supplementary information). Briefly, for Western blotting, protein samples (equivalent to 1.6 to 3.3 × 10^6 parasites) were loaded onto precast 4–12% Bis-Tris NuPAGE gels, separated by electrophoresis and transferred onto nitrocellulose membrane. Membranes were then probed with mouse anti-GFP (1:1000) or rabbit anti-PfNT1 (1:1000) primary antibodies. Horseradish Peroxidase (HRP)-conjugated anti-mouse and anti-rabbit secondary antibodies were both used at 1:10 000 dilution. Additional detail is provided in Supplementary information.

2.6. Quantitative real time PCR

Total parasite RNA was purified using RNeasy Mini Kit (QIAGEN) according to the manufacturer's protocol for purification of total RNA from animal cells. cDNA was synthesised from parasite total RNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative real time PCR (qRT-PCR) were performed using the LightCycler 480 Instrument (Roche) with *ornithine aminotransferase* [24] designated as the housekeeper gene (Supplementary information). The primers used for amplification are shown in the Supplementary information. The level of *PfHK* transcript relative to that of the housekeeper gene was calculated as previously described [25].

2.7. SYBR green I-based growth assays

The in vitro antiplasmodial activity of compounds was investigated using the SYBR green I-based fluorescence assay [26], with minor modifications (Supplementary information).

2.8. Parasite accumulation of phospho-[^{14}C]2-DG

The accumulation of phospho-[^{14}C]2-DG into saponin-isolated trophozoite stage parasites was measured as described previously [16]. The data from the phospho-[^{14}C]2-DG accumulation experiment were expressed as "distribution ratio" – the concentration of radiolabelled solute inside the cell ($[CPM]_{in}$) relative to the concentration of [^{14}C]2-DG in the extracellular solution ($[CPM]_{out}$). Distribution ratios were calculated using a trophozoite intracellular water volume of 28 femtoliters [20].

2.9. Phosphorylation of [^{14}C]2-DG by parasite lysate

[^{14}C]2-DG or [^{14}C]pantothenate (included as a control) phosphorylation by lysates prepared from saponin-isolated trophozoite stage parasites was assayed using the Somogyi reagent (combination of $ZnSO_4$ and $Ba(OH)_2$) as described recently [27]. The phosphorylation rate was determined by calculating the gradient of the linear extrapolations of the curves either between the 1 and 4 min time points ([^{14}C]2-DG) or the entire time course ([^{14}C]pantothenate).

2.10. Statistical analysis

Statistical significance was determined using two-tailed or one-tailed Student's *t* tests for paired or unpaired samples, as appropriate. When the means of more than two populations were compared, one-way analysis of variance (ANOVA) was performed. Pairwise comparisons were made post hoc with Tukey's multiple comparisons test. Regression analysis was carried out using SigmaPlot for Windows version 11.0 (Systat Software, Inc). Unless otherwise indicated, error values represent S.E.M.

3. Results

Previous attempts to localise PfHK have presented contradictory results [12]. In this study, the native *PfHK* coding sequence (Gene ID: PF3D7_0624000; [11]), without its stop codon, was cloned into pGlux-1, directly upstream of a GFP coding sequence and transfected into wild-type (3D7 strain) *P. falciparum*. To determine the proportion of fluorescent parasites in the 3D7-PfHK^{GFP} population, FACS analysis was performed. On average, 95 ± 2% (N = 5) of the parasites in the 3D7-PfHK^{GFP} line were fluorescent (Fig. 1A). To elucidate the intracellular location of PfHK, wet mounts of 3D7-PfHK^{GFP}-infected erythrocytes were observed using confocal microscopy. We show that GFP-associated fluorescence (green) was diffuse throughout the parasite cytosol, both in the ring and trophozoite stages (Fig. 1B). To confirm the predominantly cytosolic location of PfHK-GFP, we carried out Western blot analysis of the soluble and membrane-bound protein fractions of 3D7-PfHK^{GFP} trophozoites (Fig. 1C). Protein samples from whole parasites were also loaded as controls. Blots treated with anti-GFP antibody indicate the presence of a single polypeptide band of ~83 kDa for whole parasite and soluble protein fraction samples, which corresponds to the predicted mass of the PfHK-GFP fusion protein. The small amount of PfHK-GFP observed in the membrane fraction is likely due to contamination of this fraction by cytosolic proteins, as observed previously for other soluble proteins such as GFP [28]. In control blots treated with an antibody against Nucleoside Transporter 1 (PfNT1 – an integral membrane protein), a ~36 kDa band was detected (within the range of previous reports [29,30]) in whole parasite and membrane fraction samples, and could not be detected in the soluble protein fraction. Therefore, under the conditions of our experiments, PfHK appears to be cytosolic and is not associated with the PPM as suggested previously [12].

In order to characterise PfHK further, we generated a cell line, "3D7-PfHK^{+}", overexpressing the native PfHK. A parasite line, "3D7-Plasmid", harbouring an empty pGlux-1 plasmid, was also generated and used as a control. Using SYBR green I-based qRT-PCR, we show (Fig. 2A) that 3D7-PfHK^{+} parasites have a 6 ± 2-fold higher (P = 0.038) level of *PfHK* transcript relative to 3D7-Plasmid, with both normalised to the housekeeper mRNA (*ornithine aminotransferase*).

We then investigated the effect of *PfHK* overexpression on the parasites' sensitivity to the glycolysis-inhibiting compound 2-DG, using doxycycline (an antimalarial with a mechanism of action unrelated to glycolysis [31]) as a control compound. The data in Fig. 2B illustrate the effect of doxycycline on the proliferation of the *P. falciparum* lines tested. The doxycycline IC_{50} values (drug concentration that inhibits parasite proliferation by 50%) for 3D7 (22 ± 2 μM), 3D7-Plasmid (27 ± 4 μM), and 3D7-PfHK^{+} (24 ± 1 μM) were not significantly different from each other (P > 0.05, one-way ANOVA with Tukey's post-test). The effect of 2-DG on the proliferation of the *P. falciparum* lines is shown in Fig. 2C. The data obtained for 3D7-PfHK^{+} are shifted to the right of those obtained for the control lines. The 2-DG IC_{50} value for 3D7-PfHK^{+} (10 ± 1 mM) was approximately 2-fold higher than the values for the control lines (3D7 = 6.3 ± 0.3 mM; 3D7-Plasmid = 6.1 ± 0.2 mM). This difference reached statistical significance (P < 0.001, one-way ANOVA with Tukey's post-test). These results are consistent with *PfHK* overexpression in 3D7-PfHK^{+} conferring a protective effect against 2-DG.

To test whether *PfHK* overexpression would increase the rate of glucose phosphorylation, and hence its entry into glycolysis (*i.e.* whether PfHK activity is rate-limiting), we measured the uptake and phosphorylation of [^{14}C]2-DG in 3D7-PfHK^{+} and 3D7-Plasmid parasites. We utilised [^{14}C]2-DG instead of radiolabelled glucose because 2-DG has been shown to be transported and phosphorylated through the same processes as glucose, but does not proceed further into glycolysis [17]. The amount of radioactivity inside the cell will therefore represent the amount of 2-DG and phospho-2-DG accumulated within the parasites.

As shown in Fig. 3A, the accumulation of phospho-[^{14}C]2-DG is rapid, reaching a distribution ratio of ~20 within the first 4 min of the time course for both 3D7-Plasmid and 3D7-PfHK^{+}. This is consistent with the findings of previous reports [17,32,5,16]. The initial rates of phospho-[^{14}C]2-DG accumulation (determined from the gradient of fitted curves at time point "0") in the two cell lines (3D7-Plasmid = 14 ± 3 distribution ratio/min; 3D7-PfHK^{+} = 15 ± 1 distribution ratio/min) were not significantly different (P = 0.77). There are two main possible explanations for this result: (i) although *PfHK* is overexpressed in 3D7-PfHK^{+} (Fig. 2A), the level of PfHK activity may be regulated by the parasite and/or (ii) the rate of [^{14}C]2-DG transport into the parasite by PfHT may be limiting the rate of [^{14}C]2-DG phosphorylation within the parasite.

To distinguish between these two possibilities, we measured the rate of [^{14}C]2-DG phosphorylation by parasite lysates prepared from 3D7-PfHK^{+} and 3D7-Plasmid trophozoites. Fig. 3B shows that the phosphorylation rate of [^{14}C]2-DG by 3D7-PfHK^{+} lysate (3.0 ± 0.5 μmol/10^{12} cells/min) was approximately 3-fold higher (P < 0.01, one-way ANOVA with Tukey's post test) than

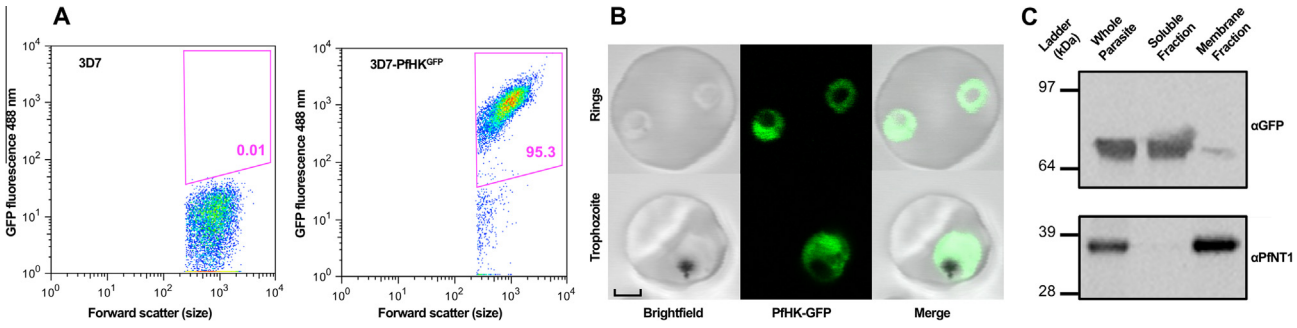

Fig. 1. (A) Representative FACS analysis of 3D7 and 3D7-PfHK^{GFP} *P. falciparum* cell lines. 3D7-PfHK^{GFP} parasites episomally express PfHK that has been tagged with GFP at the protein's carboxyl end. Expression of the fusion protein is regulated by the *P. falciparum* chloroquine resistance transporter promoter. The proportion of non-fluorescent 3D7 cells was used to establish a threshold above which cells are defined as fluorescent (represented by the pink trapezoid gates). The pink numbers indicate the percentage of fluorescent cells in the population. The data shown are from a single experiment representative of five independent experiments. (B) Localisation of GFP-tagged PfHK using confocal microscopy. Brightfield, GFP-fluorescence and merged images of erythrocytes infected with two ring-stage parasites (top) and a trophozoite-stage parasite (bottom). Scale bar represents approximately 1.5 μm. (C) Western blot analysis of 3D7-PfHK^{GFP} *P. falciparum* whole parasites (1.6 to 3.3 × 10^{6} cells) and, soluble and membrane-bound proteins from the same number (and preparation) of parasites. Nitrocellulose membranes containing samples from the same fractions were probed with either anti-GFP or anti-PfNT1 antibodies. Position and size (in kDa) of the reference ladder bands are indicated. The data shown are from a single experiment representative of two independent experiments. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

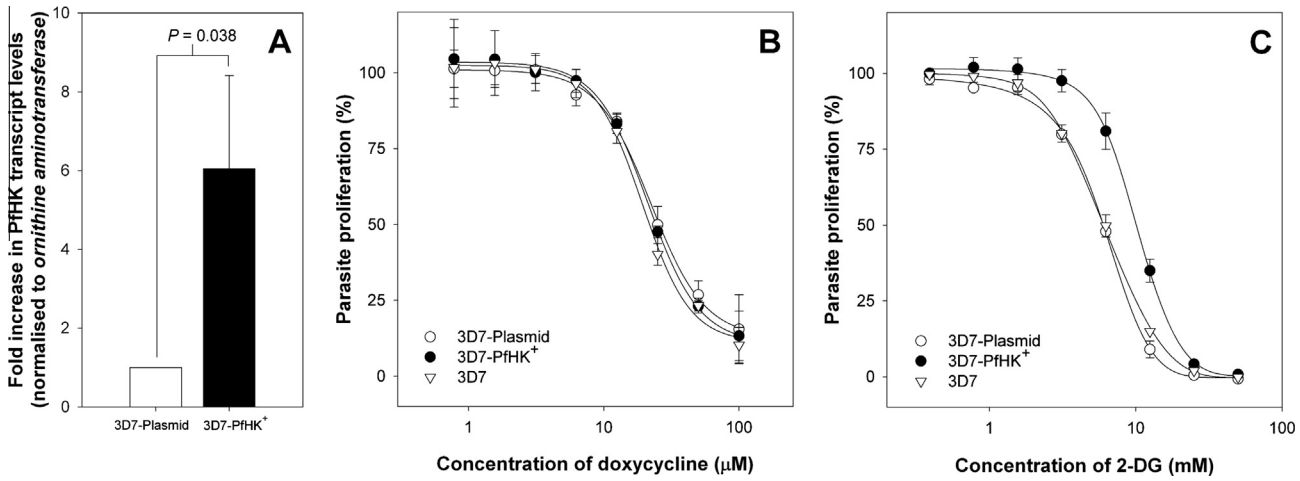

Fig. 2. (A) Quantitative real-time PCR analysis of *PfHK* expression normalised to *ornithine aminotransferase* in 3D7-Plasmid (white bar) and 3D7-PfHK$^+$ (black bar) trophozoites. The data are averaged from four independent experiments, each performed in triplicate. The *P*-value was obtained from a one-tailed, unpaired Student's *t*-test. The in vitro antiplasmodial activities of (B) doxycycline and (C) 2-deoxy-D-glucose against 3D7-Plasmid (open circles), 3D7-PfHK$^+$ (closed circles) and 3D7 (open triangles) *P. falciparum* lines. The data are averaged from three and five independent experiments, respectively, each performed in triplicate. Error bars represent S.E.M. and where not shown are smaller than the symbols.

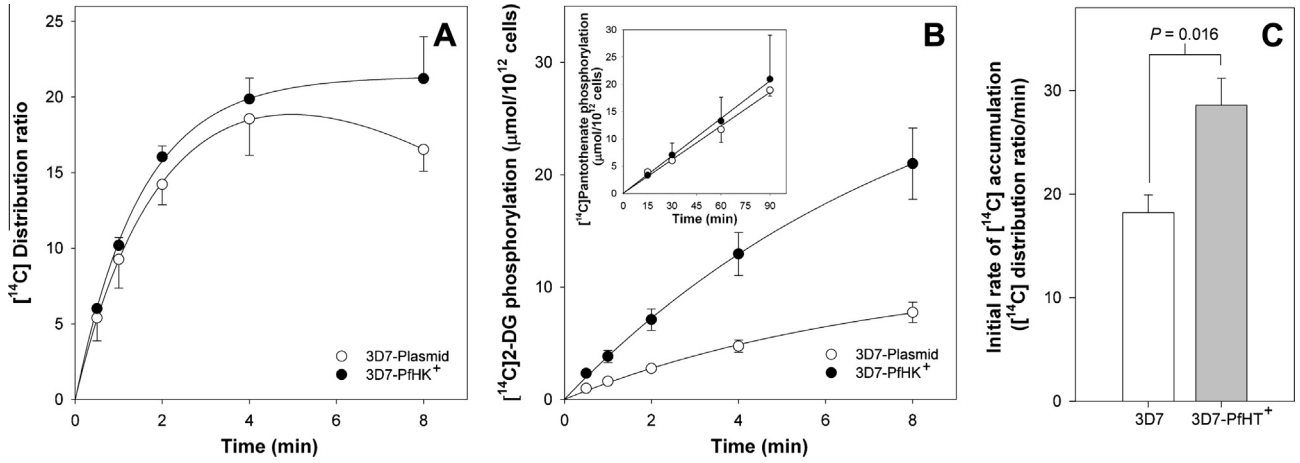

Fig. 3. (A) The accumulation of phospho-[^{14}C]2-deoxy-D-glucose in isolated 3D7-Plasmid (open circles) and 3D7-PfHK$^+$ (closed circles) *P. falciparum* trophozoites suspended in standard malaria saline containing 0.2 mM D-glucose. The data are averaged from three independent experiments, each performed in duplicate. (B) The phosphorylation of [^{14}C]2-deoxy-D-glucose and [^{14}C]pantothenate (inset) by lysates prepared from 3D7-Plasmid and 3D7-PfHK$^+$ trophozoites. The data are averaged from five and two independent experiments respectively, each performed in duplicate. The [^{14}C]pantothenate phosphorylation experiments were paired (*i.e.* using the same batch of lysates) with two of the five [^{14}C]2-deoxy-D-glucose experiments. (C) The initial rate of accumulation of phospho-[^{14}C]2-deoxy-D-glucose by trophozoites isolated from the 3D7 (white bar) and 3D7-PfHT$^+$ (grey bar) parasite lines. The data are averaged from four independent experiments, each performed in duplicate. The *P*-value was obtained from a two-tailed, unpaired Student's *t*-test. For all of the figures, error bars represent S.E.M. except for the inset where they represent range/2. Where not shown, error bars are smaller than the symbols.

that by 3D7-Plasmid lysates (1.0 ± 0.1 μmol/10^{12} cells/min). In comparison, the phosphorylation rates of [^{14}C]pantothenate (included as a control) by lysates isolated from 3D7-Plasmid (0.21 ± 0.02 μmol/10^{12} cells/min; mean ± range/2) and 3D7-PfHK$^+$ (0.23 ± 0.08 μmol/10^{12} cells/min; mean ± range/2) did not differ markedly from each other (Fig. 3B inset). This result with pantothenate, therefore, confirms that the observed increase in the [^{14}C]2-DG phosphorylation rate by 3D7-PfHK$^+$ is not due to elevated total protein/enzyme quantity between the control and PfHK overexpressing cell lines. These results are consistent with the 3D7-PfHK$^+$ line having the capacity for increased PfHK activity and support the possibility that PfHT limits the rate of glucose entry into the parasite and, consequently, its phosphorylation. To investigate this directly, we determined the initial rate of phospho-[^{14}C]2-DG accumulation in 3D7 and 3D7-PfHT$^+$, a previously generated transgenic *P. falciparum* line shown to overexpress *PfHT* by approximately 3-fold [33]. Fig. 3C illustrates the 58 ± 9% higher initial rate of accumulation of phospho-[^{14}C]2-DG ($P = 0.016$) in 3D7-PfHT$^+$ compared with 3D7. This supports the hypothesis that the rate of glucose transport by PfHT limits the rate of glucose entry into glycolysis in *P. falciparum*.

4. Discussion

4.1. PfHK localisation

PfHK has been reported to possess a potential hydrophobic membrane anchor sequence at its carboxyl terminus [10]. The mammalian Type I hexokinase isozyme also possesses a hydrophobic sequence at its amino terminus, which has been shown to be essential for its binding to the mitochondrial outer membrane [34]. Based on this similarity, Olafsson and Certa conducted immunoelectron microscopy experiments using anti-PfHK antibodies and concluded that PfHK may be associated with the PPM [12]. A

separate study characterising the hexokinase of *Toxoplasma gondii* also found a similar hydrophobicity profile in the hexokinase carboxyl terminus that is thought to explain the enzyme's ability to associate weakly with the membrane [35]. It was argued that such an arrangement would lead to more efficient glucose accumulation; glucose molecules taken up by the parasites via the glucose transporter would immediately be phosphorylated by the nearby hexokinase and thereby funnelled into glycolysis. However, some experiments in both investigations [12,35] indicated that the majority of hexokinase was located in the cytosol of these parasites.

In this study, a transgenic parasite line that expresses a GFP-labelled PfHK was generated to investigate further the enzyme's location within *P. falciparum*. It was observed that the GFP fluorescence was diffuse throughout the cytosol of the transgenic parasite in both the ring and trophozoite stages, indicating a mainly cytosolic location of PfHK (Fig. 1B). Additionally, from the Western blot analysis performed here, the PfHK-GFP polypeptide was detected almost exclusively in the cytosolic parasite fraction (Fig. 1C). There are several examples of other *P. falciparum* encoded proteins that have been successfully localised to their predicted targets using the GFP reporter system used here [36], including a GFP-tagged protein (glideosome-associated protein 50) which, like the PfHK-GFP construct used here, contains a transmembrane domain located just upstream of the GFP label at the carboxyl terminus [37]. Furthermore, previous affinity chromatography experiments involving parasite cytosolic fractions (derived from supernatants of parasite lysates centrifuged at $100000\times g$ to remove membranes), captured PfHK using immobilised baits [38,39]. This is consistent with PfHK being a cytosolic protein, as observed in this study.

4.2. Drug sensitivity of 3D7-PfHK$^+$

Overexpression of target proteins is frequently utilised as a strategy to validate potential drug targets [40]. For example, it was shown in a study that overexpression of a leucyl aminopeptidase in a transgenic *P. falciparum* strain reduced the parasite's sensitivity to bestatin by 2.5-fold when compared with the parent strain [41]. Using the same approach, a parasite line overexpressing PfHT (the strain used in this study) was reported to be 2.5-fold less sensitive to the PfHT-targeting inhibitor, compound 3361, than wild-type parasites [33]. The observation in this study that parasites overexpressing PfHK are almost 2-fold less sensitive (Fig. 2C) to the antiplasmodial activity of 2-DG (a known glycolysis inhibitor) when compared with wild-type parasites and parasites containing an "empty" vector, is consistent with PfHK being a potential drug target. The demonstration that parasite sensitivity to doxycycline (which has a mode of action unrelated to glycolysis [31]) is unchanged when PfHK is overexpressed, supports the hypothesis that the shift in 2-DG sensitivity is specifically a result of PfHK overexpression. It has been previously shown that 2-DG significantly inhibits *P. falciparum* growth in vitro, most likely via an effect on parasite glycolysis [16]. Therefore, the data generated in the present study offer direct support of this hypothesis. The exact mechanism through which 2-DG inhibits parasite growth, however, remains to be fully understood. In *P. falciparum*, it was determined that 2-DG enters the parasite mainly through PfHT [3], and is phosphorylated by PfHK to phospho-2-DG, which accumulates within the parasite because it does not proceed further within the glycolytic pathway [17]. A likely explanation, therefore, would be that phospho-2-DG mediates parasite growth inhibition. In mammalian cells, 2-DG has likewise been observed to accumulate in the form of phospho-2-DG [42]. It was also suggested that phospho-2-DG inhibits the enzyme phosphohexoisomerase and that this is the primary mechanism by which it blocks glycolysis [42]. However, more recent findings show that phospho-2-DG exerts a secondary non-competitive inhibitory effect on hexokinase [43]. Although it is possibly similar in nature to the allosteric inhibition of the enzyme by glucose-6-phosphate, the concentration of phospho-2-DG required for this purpose is much higher [43], which may explain the apparent absence of hexokinase inhibition by phospho-2-DG in previous studies [44].

Based on these observations, the decreased 2-DG sensitivity in parasites overexpressing PfHK (Fig. 2C) is consistent with phospho-2-DG inhibiting PfHK. The glucose analogue 2-FG, another inhibitor of *P. falciparum* growth [16], was postulated to inhibit mammalian hexokinase in the same way as glucose-6-phosphate after it is phosphorylated to phospho-2-FG [45]. Furthermore, a recent report has shown that glucose-6-phosphate inhibits heterologously-expressed PfHK [46]. From the observations in these studies, phospho-2-DG is likely to inhibit the *P. falciparum* hexokinase in a way that is similar to glucose-6-phosphate regulation. It should be noted that the 3D7-PfHK$^+$ parasite line generated here has the potential of being used in future studies to confirm the mechanism of action of drugs that are designed to target, or are suspected of targeting, PfHK.

4.3. Checkpoint for glucose entry into glycolysis

Prior to this study, it was not known whether PfHK overexpression would result in increased hexokinase activity or whether the additional PfHK would be regulated to maintain hexokinase activity at a certain level. To resolve these possibilities, we measured and compared the rates of [^{14}C]2-DG accumulation and phosphorylation by wild-type and PfHK overexpressing cell lines. It was observed that there was no difference in the accumulation rates of phospho-[^{14}C]2-DG between the saponin-isolated 3D7-PfHK$^+$ and 3D7-Plasmid parasites (Fig. 3A). The [^{14}C]2-DG phosphorylation rate by lysates prepared from 3D7-PfHK$^+$ parasites was, however, significantly higher (by 3-fold) than the rate determined from lysates prepared from 3D7-Plasmid parasites (Fig. 3B).

These observations can be explained simply by the transport of glucose across the PPM being rate-limiting for glucose entry into glycolysis. The rate of glucose transport into the parasite by PfHT could be slower than that of PfHK-catalysed glucose phosphorylation. If this is the case, overexpression of PfHK will not cause a corresponding increase in the amount of glucose-6-phosphate generated. Here, we observe that overexpression of PfHT (which would increase the rate of glucose transport into the parasite) resulted in a 1.5-fold increase in the rate of [^{14}C]2-DG accumulation. As 2-DG accumulation requires the coordinated action of PfHT and PfHK, it is consistent with PfHT transport being rate-limiting for glucose entry into glycolysis. Studies involving the bloodstream form of *Trypanosoma brucei* (another protozoan parasite) have also suggested that glucose transport is most likely the rate-limiting step of this parasite's glycolytic pathway [47].

In conclusion, we show that in contrast to some previous suggestions, PfHK is located within the cytosol of *P. falciparum*. The overexpression of PfHK reduced the parasite susceptibility to 2-DG, consistent with PfHK being the target of its antiplasmodial activity. This finding supports the notion that glycolysis is a viable antimalarial drug target. Furthermore, we present evidence consistent with glucose transport by PfHT limiting the rate of glucose entry into *P. falciparum* glycolysis, supporting the suitability of PfHT as a drug target.

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Appendix A. Supplementary information

Supplementary information associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2013.07.052.

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